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O,5′-Thymidylyl-L-serine: A Model for Possible Linkers in DNA*

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ABSTRACT: *O*,5′-Thymidylyl-L-serine, a model for possible linkers in deoxyribonucleic acid (DNA), was prepared by two synthetic methods. This compound was unaffected by a wide variety of exo- and endonucleases or peptidases, as well as various chemical agents, such as hydroxylamine and sodium hyponitrite. However, it was cleaved into serine and thymidylic acid by snake venom phosphodiesterase. These properties should prove useful in attempts to identify such linkers in DNA.

Discussion

The proposal (Bendich and Rosenkranz, 1963) that seryl (or threonyl) oligopeptides (I, Ia, Ib) may serve as linkers or "punctuation" in the genetic code of deoxyribonucleic acids (DNA) prompted the synthesis of model compounds for aid in the identification of such fragments in partial hydrolysates of DNA. The proposal was based, in part, on the isolation of amino acids, including *O*-phosphoserine, from mineral acid hydrolysates of rigorously deproteinized DNA's from mammalian, bacterial, and viral sources and the fragmentation of DNA by hydroxylamine derivatives and alkali (Borenfreund *et al.*, 1961; Bendich and Rosenkranz, 1963; Bendich *et al.*, 1964; and references cited therein). The natural occurrence of amino acids in DNA appears to be widespread (Balis *et al.*, 1964; Champagne *et al.*, 1964; Olenick and Hahn, 1964).

The carboxylic ester linkage in II is analogous to the bond uniting the amino acid or growing peptide chain to the 3′- (or 2′-) hydroxyl of the terminal adenosine in transfer RNA's (Hoagland *et al.*, 1958; Zachau *et al.*, 1958; Hecht *et al.*, 1959; Zachau, 1960; Zachau and Karau, 1960; Nathans *et al.*, 1962; Feldman and Zachau, 1964). The serine nucleotide shown in III has not yet, to our knowledge, been found in nature. Since serine and threonine constituted about one-third of the amino acids isolated from the DNA of bull sperm (Borenfreund *et al.*, 1961), it was suggested that the seryl peptide was a small one, containing perhaps only a few amino acids (Ib).

The identification of fragments similar to II or III in appropriate partial hydrolysates of DNA would constitute persuasive evidence for the existence of such linkages within nucleic acids. To this end, a knowledge of the properties of such possible fragments of known structure would be helpful. Efforts were directed toward the synthesis of nucleotide derivatives similar to III since they would be expected to be more stable than those of type II. For instance, hydroxylamine or mild alkali would be expected to hydrolyse II but not III (Zachau, 1960; Zachau and Karau, 1960; Sokolova *et al.*, 1962). The phenylalanyl ester of thymidine (II, R = benzyl) has been prepared (Sokolova *et al.*, 1963) and found to cleave readily with hydroxylamine.

Results

Two straightforward synthetic routes (Scheme I) to *O*,5′-thymidylyl-L-serine (IV) were attempted, and both led to the desired product. (A) Adaptation of the dicyclohexylcarbodiimide phosphodiester synthesis of Khorana using *N*-carbobenzyloxy-L-serine benzyl ester

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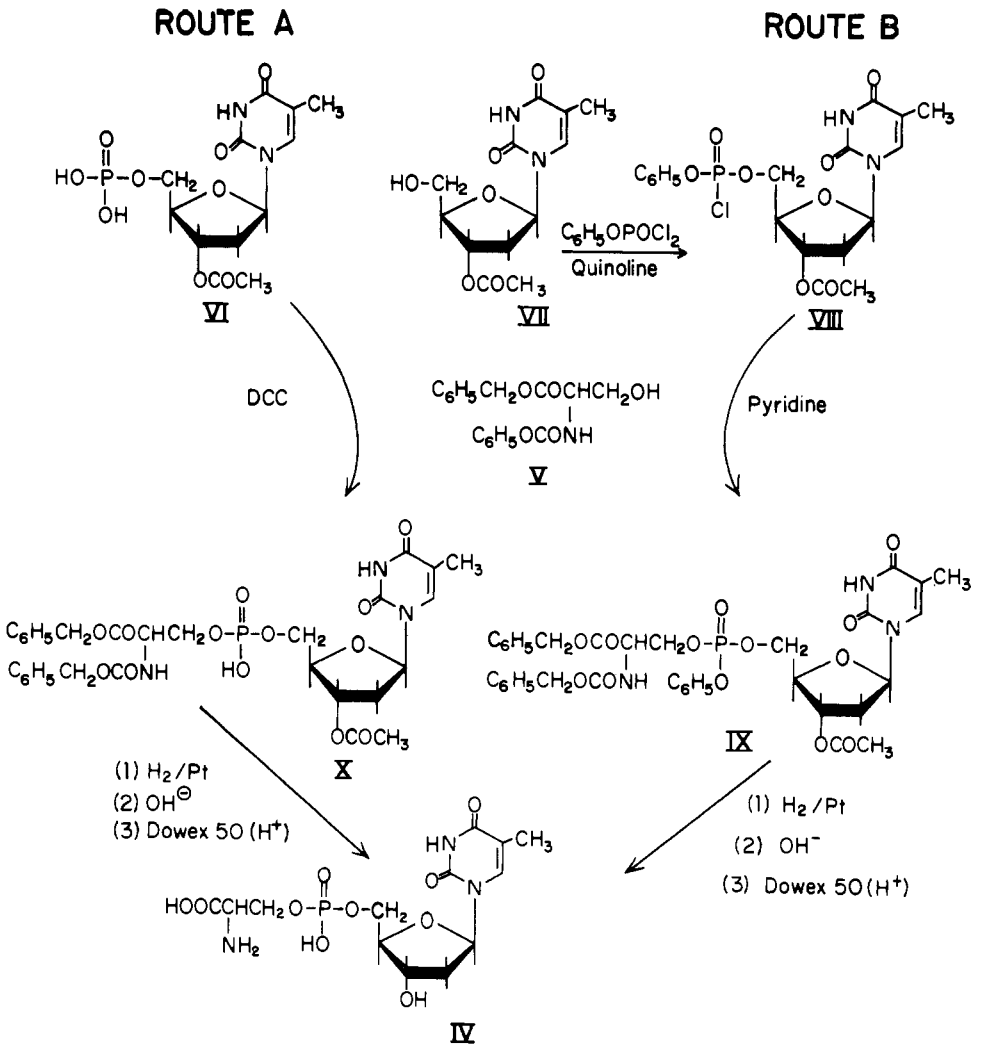


SCHEME I

TABLE I: Ultraviolet Spectra.

	5′-Thymidylic Acid			*O*,5′-Thymidylyl-L-serine (IV)	
	λ_{max} (ϵ)	λ_{min} (ϵ)		λ_{max} (ϵ)	λ_{min} (ϵ)
pH 2.0	267 (9600)	233 (2300)	pH 2.9	267 (9200)	234 (1700)
pH 7.0	267 (9600)	234 (2400)	pH 6.5	267 (9200)	234 (1700)
pH 12.0	267 (7800)	243 (4500)	pH 11.4	267 (7400)	244 (2700)

(V) and 3′-*O*-acetyl-5′-thymidylic acid (VI) afforded diester IV in a yield of 38% (Gilham and Khorana, 1958). (B) The approach of Baer for the synthesis of various glycerylphosphorylserine derivatives was adapted to the preparation of IV in 17% yield from V and 3′-*O*-acetylthymidine (VII) (Baer *et al.*, 1959; Baer and Maurukas, 1955). The conditions for optimum yields were not investigated for either route.

Some of the ultraviolet spectral and chromatographic properties of *O*,5′-thymidylyl-L-serine (IV) are listed in Tables I and II. Data relating to the identity of IV are summarized in the Experimental Section.

The stability of thymidylylserine toward various agents potentially useful for degrading DNA was studied. Dilute mineral acid (0.5 N hydrochloric acid, 160 min at 100°) hydrolyzed compound IV to a mixture of thymidine, thymidylic acid, serine, and phosphoserine; starting material was also recovered.

TABLE II: Paper Chromatographic and Electrophoretic Properties.[a]

	Solvent		
	A (R_F)	B (R_F)	C (cm/hr.)
O,5′Thymidylyl-L-serine (IV)	0.25	0.58	3.8
Thymidine	0.64	0.75	−0.1
5′-Thymidylic acid	0.35	0.52	4.9
Serine	0.30	0.65	−1.0
O-Phosphoserine	0.19	0.44	5.6

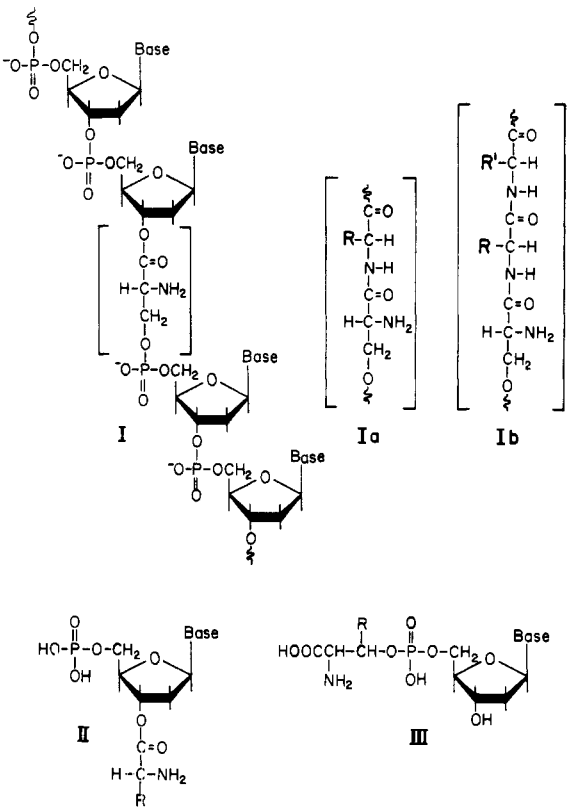
[a] Solvent A: 1-butanol–water–acetic acid, 4:2:1; Whatman No. 1 paper; B: 2-propanol–water–concentrated ammonia, 3:2:1; Whatman No. 1 paper; C: 0.2 M acetate buffer, pH 3.2; 16.5 v/cm; Whatman No. 3 MM paper.

The phosphate–serine bond seemed to hydrolyze somewhat faster than the phosphate–thymidine bond. Of the various endo- and exonucleases and phosphodiesterases studied (Table III) only snake (*Crotalus adamanteus*) venom phosphodiesterase, well known for its specific 5′-phosphodiesterase activity, degraded the compound (into serine and 5′-thymidylic acid). A variety of peptidases and other chemical agents examined had no effect on the compound (see Table III). Thus, should a fragment such as thymidylylserine (IV) form part of the main chain of DNA, it should be possible to release it by treatment with DNAase and a variety of appropriate phosphodiesterases (excluding snake venom phosphodiesterase) and peptidases. Such studies are in progress.

Experimental Section

The spectrophotometric measurements were made with a Cary Model 11 recording spectrophotometer. Elemental analyses were carried out by the Schwarzkopf Microanalytical Laboratory, New York, N. Y.

O,5′-Thymidylyl-L-serine. Method A. A solution of 412 mg (1.0 mmole) of 3′-*O*-acetyl-5′-thymidylic acid (VI) (Gilham and Khorana, 1958), 3.294 g (10.0 mmoles) of *N*-carbobenzyloxy-L-serine benzyl ester (V) (Cyclo Chemical Corp., recrystallized), and 5.158 g (25.0 mmoles) (Tomlinson and Tener, 1964) of dicyclohexylcarbodiimide in 50 ml of anhydrous pyridine was kept at room temperature for about 40 hr. The disappearance of acetylthymidylic acid was followed by thin-layer chromatography on silica gel using 2-propanol–water–concentrated ammonia, 7:2:1. The reaction mixture was diluted with 110 ml of water and stirred at room temperature for 1 hr, extracted repeatedly with ether, acidified by stirring with Dowex 50 resin (H^+ form), and finally concentrated under reduced pressure to give 306 mg of crude diester X.



This was not characterized further and was subsequently hydrogenated.

HYDROGENATION OF DIESTER X. Crude diester X, 306 mg (*ca.* 0.59 mmole), was hydrogenated at atmospheric pressure and room temperature over 21 mg of platinum oxide (Adam's catalyst) in 35 ml of 30% ethanol acidified with acetic acid. Palladium on charcoal (5%; two fresh 30-mg portions) was ineffective. Hydrogenation (in 20 ml of 50% ethanol over 50 mg of platinum oxide) of 396 mg of crude diester X similarly prepared required 21 ml (73% of theory) of hydrogen gas before uptake ceased after 18 hr. (A sodium hydroxide trap was included in the system to remove the carbon dioxide liberated.)

The hydrogenation mixture was diluted with 50 ml of water, adjusted to pH 12 with 20% sodium hydroxide, and warmed for 15 min on a steam bath (to remove the acetyl group). The hydrolysate was acidified by treatment with Dowex 50 resin (H^+ form), extracted three times with ether, and finally concentrated under reduced pressure to give 217 mg (53%) of crude diester IV as an amorphous white powder. The crude material was purified by chromatography on Whatman No. 1 paper in solvent system A (described below). (The product band was identified by the concurrence of ultraviolet absorption and a positive ninhydrin color reaction.) Such purification of 32 mg of crude material gave 23 mg (38%) of thymidylylserine (IV) as an amorphous white powder which could not be induced to crystallize. Chromatographic and spectral characteristics are summarized in Tables I and II. The compound exhibited $[\alpha]_D^{24}$ +28 ± 2° (H_2O, pH 6.5).

TABLE III: Hydrolytic Studies.[a]

Agent	Minimum Concentration	Buffer	pH	Salts	Special additions	Temp., °C.	Substrate, M (IV)
Snake venom phosphodiesterase[b]	400 μg/ml[c]	0.04 M Tris[d]	8.8	0.01 M $MgCl_2$		37	0.0049
Deoxyribonuclease I (pancreas)	80 μg/ml	0.10 M Tris	7.0	0.045 M $MgCl_2$ 0.0045 M $CaCl_2$		37	0.0059
Deoxyribonuclease II (spleen)	800 μg/ml	0.05 M Ac^-[e]	4.6	0.05 M $MgCl_2$		37	0.0059
E. coli phosphodiesterase I	176 U/ml[f]	0.066 M gly[g]	9.4	0.001 M $MgCl_2$ 0.015 M NaCl		37	0.0059
E. coli phosphodiesterase III	104 U/ml	0.03 M Tris	8.0	0.01 M $MgCl_2$	0.008 M β-mercaptoethanol	37	0.0059
Streptodornase (strep.)	200 μg/ml[c]	0.10 M Tris	7.2	0.0036 M $MgCl_2$		37	0.0059
				0.0036 M $CaCl_2$		37	0.0059
Micrococcal deoxyribonuclease	200 μg/ml	0.05 M Tris	8.6	0.0045 M $CaCl_2$		37	0.0059
Ribonuclease	100 μg/ml	0.01 M Tris	7.2	0.00036 M $MgCl_2$ 0.00036 M $CaCl_2$		37	0.0059
Leucine aminopeptidase	34 U/ml	0.10 M Tris	8.5	0.005 M $MgCl_2$		31	0.0245
Carboxypeptidase	10 U/ml	0.10 M Tris	7.5	0.10 M NaCl		31	0.0245
Pepsin	11.5 U/ml	0.01 M HCl	2.1			31	0.0245
α-Chymotrypsin	9.1 U/ml	0.10 M Tris	8.0	0.05 M $CaCl_2$ 0.25 M NaCl		31	0.0245
Subtilopeptidase	10 U/ml	0.10 M PO_4[h]	8.0	0.20 M NaCl		31	0.0245
Papain	100 U/ml	0.10 M Tris	6.0	0.005 M NaCl	0.005 M EDTA[i]	31	0.0245
					0.005 M cysteine	31	0.0245
Hydroxylamine	0.067 M	0.10 M Tris	7.0			31	0.0245
Hydroxyurea	0.067 M	0.10 M Tris	7.0			31	0.0245
Sodium Hyponitrite	0.067 M	0.10 M Tris	7.0			31	0.0245

[a] All incubations were carried out for a minimum of 48 hr. [b] *Crotalus adamanteus*. [c] These enzyme preparations were not standardized but were shown to be active in degrading DNA at these concentrations. [d] Tris(hydroxymethyl)-aminomethane. [e] Ac^- = acetate–acetic acid. [f] U = international enzyme units = μmole/min. [g] gly = glycine. [h] 0.20 M NH_4Cl + 0.10 M Na_2HPO_4. [i] Ethylenediaminetetraacetic acid, disodium salt.

Method B. A solution at room temperature of 562 mg (1.98 mmoles) of 3′-*O*-acetylthymidine (VII) (Michelson and Todd, 1953) and 1.00 g (7.78 mmoles) of redistilled anhydrous quinoline in 2.5 ml of chloroform (dried over anhydrous calcium sulfate) was added in the absence of moisture, with vigorous stirring, during 25 min to 416 mg (1.98 mmoles) of cold (ice-salt) phenylphosphorodichloridate (Eastman Kodak). To this solution was added 0.9 ml of anhydrous pyridine and stirring was continued for 30 min in the cold and then for 30 min at room temperature.

The pink reaction mixture was rechilled (ice–salt) and a solution of 659 mg (2.0 mmoles) of *N*-carbobenzyloxy-L-serine benzyl ester (V) in 2.3 ml of anhydrous pyridine was added in one portion. The resulting solution was stirred at 10–20° for 2 hr and at room temperature for 20 hr. During this time, the reaction mixture turned from a pale straw color to a deep purple.

The reaction mixture was taken up in chloroform, washed successively with 5% hydrochloric acid, saturated aqueous sodium bicarbonate, and water, dried over magnesium sulfate, and finally concentrated under reduced pressure to give 1.23 g of crude material. Chromatography of the crude neutral product on silica gel, with ethyl acetate as the final eluent, afforded 290 mg (19%) of triester IX as a colorless glass which could not be induced to crystallize.

Anal. Calcd for $C_{36}H_{38}N_3O_{13}P$: C, 57.52; H, 5.10; N, 5.59; P, 4.12. Found: C, 57.46; H, 5.45; N, 5.67; P, 3.96.

Crude triester IX (121 mg) was hydrogenated over 50 mg of platinum oxide in about 25 ml of 99% ethanol giving, after hydrolysis and acidification, 63 mg of crude diester IV. Chromatography of 41 mg of this material as before gave 36 mg (17%) of amorphous white powder identical in ultraviolet spectrum, ninhydrin test, and paper chromatographic and electro-

phoretic properties with diester IV afforded by Method A.

The structure of IV was confirmed by the following evidence: (1) Chromatographic examination of a partial hydrolysate (0.5 N hydrochloric acid at 100° for 160 min) revealed the presence of starting material, serine, phosphoserine, thymidine, and thymidylic acid. (2) The ultraviolet absorption spectrum of the material was the same as that of thymidylic acid, as shown in Table I. (3) Hydrolysis of thymidylylserine for 9 min in boiling 70% perchloric acid released 1.00 ± 0.02 mole of inorganic phosphate (King, 1932) for each mole of thymidine (estimated by ultraviolet). (4) The ninhydrin color coefficient (Stein–Moore analyser) of the compound was 19.1 compared with 21.8 for phosphoserine and 20.3 for serine. An hydrolysate of IV (refluxed in 6 N hydrochloric acid for 24 hr) contained 0.8 mole of serine. Loss of serine under such conditions of hydrolysis has been reported previously (Soejima, 1955; Olenick and Hahn, 1964). (5) Snake venom phosphodiesterase catalyzed the cleavage of the compound into serine and 5′-thymidylic acid. The cleavage was complete under the conditions specified in Table III.

Hydrolytic Studies. Examination of the action of various enzymes and chemical agents on thymidylylserine (IV) revealed the compound to be unchanged after treatment with a wide range of phosphodiesterases, nucleases, and peptidases, with the exception of venom phosphodiesterase, noted above. In addition, the compound was not affected by hydroxylamine, hydroxyurea, or sodium hyponitrite, agents which are known to induce a limited degradation of DNA (Bendich *et al.*, 1964). Furthermore, the compound was not affected by β-mercaptoethanol and only slowly by water at pH values ranging from 4.6 to 9.4 at 37°. These compounds and enzymes and the conditions of the studies are tabulated in Table III.

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